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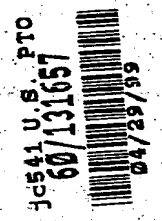

Transmitted herewith for filing is a PROVISIONAL APPLICATION of:

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for **INTRODUCTION TO THE THEORY OF FLUORESCENCE INTENSITY DISTRIBUTION ANALYSIS**. The application comprises a 17-page specification.

Accompanying this application for filing is:

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INTRODUCTION TO THE THEORY OF FLUORESCENCE INTENSITY DISTRIBUTION ANALYSIS

1. Introduction

The primary data of a fluorescence correlation experiment is a sequence of photon counts detected from a microscopic sample volume. An essential attribute of the fluorescence correlation analysis is the calculation of the second order autocorrelation function of photon detection. This is a way how a stochastic function (of photon counts) is transformed into a statistical function having an expected shape, serving as a means to estimate some parameters of the sample. However, the calculation of the autocorrelation function is not the only way for extracting information about the sample from the sequence of photon counts. Another approach, based on collecting the distribution of the number of photon counts per a given time interval, was introduced in fluorescence fluctuation spectroscopy by Qian and Elson in 1990.[i]

The idea behind the fluorescence intensity distribution analysis (FIDA) can be well understood by imagining an ideal case when a sample volume is uniformly illuminated and when there is almost never more than a single particle illuminated at a time, similar to the ideal situation in cell sorters. Under these circumstances, each time when a particle enters the sample volume, fluorescence intensity jumps to a value corresponding to the brightness of a given type of particles. Naturally, the probability that this intensity occurs at an arbitrary time moment equals the product of the concentration of a given species and the size of the sample volume. Another fluorescent species which may be present in the sample solution produces intensity jumps to another value characteristic of this other species. In summary, the distribution of light intensity is in a straightforward way determined by the values of concentration and specific brightness[ii] of each fluorescent species in the sample solution.

In reality, the intensity of fluorescence detected from a particle within a sample volume is not uniform but depends on the coordinates of the particle with respect to the focus of the optical system. Even though the calculation of a theoretical distribution of the number of photon

counts is more compl x for a bell-shaped profile than for a rectangular one, the distribution of the number of photon counts sensitively depends on values of the concentration and the specific brightness of fluorescent species. The measured distributions of the number of photon counts can therefore be used for sample analysis.

The first successful realization of this kind of analysis was demonstrated on the basis of moments of the photon count number distribution.[iii] The k-th factorial moment of the photon count number distribution $P(n)$ is defined as

$$F_k = \sum_n \frac{n!}{(n-k)!} P(n). \qquad (1)$$

In turn, factorial moments are closely related to factorial cumulants,

$$F_k = \sum_{l=0}^{k-1} C_l^{k-1} K_{k-l} F_l, \qquad (2)$$

or

$$K_k = F_k - \sum_{l=1}^{k-1} C_l^{k-1} K_{k-l} F_l. \qquad (3)$$

(C_l^k s are binomial coefficients, and K_ks are cumulants.) The basic expression used in moment analysis, derived for ideal solutions, relate k-th order cumulant to concentrations (c_i) and specific brightness values (q_i)

$$K_k = \chi_k \sum_i c_i (q_i T)^k. \qquad (4)$$

Here, χ_k is the k-th moment of the relative spatial brightness profile $B(r)$:[iv]

$$\chi_k = \int_{(V)} B^k(r) dV. \qquad (5)$$

Qian and Elson used experimental values of the first three cumulants to determine unknown paramet rs f the sample. The number of cumulants which can be reliably determin d from experiments is usually three to four. This sets a limit to the applicability f the mom nt analysis.

In the pioneer publications on moment analysis, the idea that the count number distribution could be directly fitted was also discussed. The present chapter aims at presenting an adequate theory which has found a number of applications in biochemical assay development and drug screening.

2. Photon count number distribution corresponding to a rectangular sample profile

A key to a successful realization of the photon count number distribution analysis is an adequate calculation of the expected count number distribution function. Let us first consider a simple theoretical case when the light intensity reaching the detector from a particle as a function of coordinates of the particle is constant over the whole active volume of the sample, and zero outside it. Also, we assume that the diffusion of a fluorescent particle is negligible during the counting interval T. In this case, the distribution of the number of photon counts emitted by a single fluorescent species can be analytically expressed as double Poissonian: the distribution of the number of particles of given species within this volume is Poissonian, and the conditional probability of the number of detected photons corresponding to a given number of particles is also Poissonian. The double Poissonian distribution has two parameters: the mean number of particles in the active sample volume, c and the mean number of photons emitted by a single particle per dwell time, qT. The distribution of the number of photon counts n corresponding to a single species is expressed as

$$P(n;c,q) = \sum_{m=0}^{\infty} \frac{c^m}{m!} e^{-c} \frac{(mqT)^n}{n!} e^{-mqT}, \qquad (6)$$

where m runs over the number of molecules in the active volume. If $P_i(n)$ denotes the distribution of the number of photon counts from species i, then the resultant distribution $P(n)$ is expressed as

$$P(n) = \sum_{\{n_i\}} \prod_i P_i(n_i)\delta(n, \sum n_i) \qquad (7)$$

This means that $P(n)$ can be calculated as a convolution of the series of distributions $P_i(n)$.

3. Photon count number distribution corresponding to an arbitrary sample profile: the convolution technique

Like in FCS, the rectangular sample profile is a theoretical model which can hardly be applied in experiments. The issue which particular shape of the sample profile $B(\mathbf{r})$ should be selected as a model will be studied later in this chapter. Here we will only study how the photon count number distribution of a single species can be calculated for a given sample profile. We may divide the sample into a great number of volume elements and assume that within each of them, the intensity of a molecule is constant. Contribution to photon count number distribution from a volume element is therefore double Poissonian with parameters cdV and $qIB(\mathbf{r})$. (Here q denotes count rate from a molecule in a selected standard position where $B = 1$, and $B(\mathbf{r})$ is the profile function of coordinates.) The overall distribution of the number of photon counts can be expressed as a convolution integral over double Poissonian distributions. Note that integration is a one-dimensional rather than a three-dimensional problem here, because the result of integration does not depend on actual positions of volume elements in respect to each other. Figuratively, we may rearrange the three-dimensional array of volume elements into a one-dimensional array, for example in the decreasing order of the value of B.

In a number of first experiments in our laboratory, the photon count number distribution was indeed fitted, using the convolution technique. Our sample model consisted of twenty spatial sections, each characterized by its volume V_j and brightness B_j. However, a convenient and much faster computation technique exists as described in the next subsection.

4. Photon count number distribution c rresponding to an arbitrary sample profile: th technique of the generating function

The formal definition of the generating function of a distribution $P(n)$ is as follows:

$$G(\xi) = \sum_{n=0}^{\infty} \xi^n P(n). \tag{8}$$

In particular, if one selects $\xi = e^{i\varphi}$, then the distribution $P(n)$ and its generating function $G(\varphi)$ are interrelated by the Fourier transform. What makes the generating function attractive in count number distribution analysis is the additivity of its logarithm: logarithms of generating functions of photon count number distributions of independent sources, like different volume elements as well as different species, are simply added for the calculation of the generating function of the combined distribution. (This is so because the transformation (8) maps distribution convolutions into the products of the corresponding generating functions.) Applying the definition (8) to formula (6) with $c \rightarrow cdV$ and $q \rightarrow qB(\mathbf{r})$, the contribution from a particular species and a selected volume element dV can be written as

$$G_i(\xi; dV) = \exp[c_i dV (e^{(\xi-1)q_i \tau B(r)} - 1)]. \tag{9}$$

Therefore, the generating function of the total photon count number distribution can be expressed in a closed form

$$G(\xi) = \exp[\sum_i c_i \int (e^{(\xi-1)q_i \tau B(r)} - 1) dV]. \tag{10}$$

Numeric integration according to Eq. (9) followed by a fast Fourier transform is to our knowledge the most effective means of calculating the theoretical distribution $P(n)$ corresponding to a given sample (i.e., given concentrations and specific brightness values of fluorescent species).

5. Sample profile models

The most widely used spatial profile model in FCS is the three-dimensional Gaussian profile with a single parameter of shape, the axial dimension ratio in longitudinal and radial directions. Logically, the first step in photon count number distribution analysis would be fitting a count number distribution obtained for single species like tetramethylrhodamine, see Fig.1. Residuals curve with open circles illustrates the fit quality obtainable with the Gaussian profile. There are large systematic deviations in residuals. What is a sufficiently flexible model for fitting FCS data has turned out to be an absolutely inflexible and inadequate model for FIDA.[vi]

A model of the sample profile which has yielded a better fit of the measured distribution $P(n)$ is Gaussian-Lorentzian (see Fig. 1, open squares), but this model still lacks flexibility. Note that according to Eq. (8), a certain function of the spatial brightness B is integrated over the volume. In other words, it is a relationship between B and V, characterizing a given spatial brightness profile in FIDA. For example, the Gaussian profile yields the relationship

$$\frac{dV}{dx} \propto \sqrt{x}, \tag{11}$$

where we have denoted $x = -\ln B$. The Gauss-Lorentzian profile yields the relationship

$$\frac{dV}{dx} \propto e^{x/4}\sqrt{\sinh\frac{x}{2}}. \tag{12}$$

Both of the relationships are inflexible, i.e., they do not provide any spatial shape parameters to adjust the theoretically calculated distribution to fit the measured data.

When looking for sufficiently flexible models to fit experimental data, we ended at the following relationship:

$$\frac{dV}{dx} \propto (x + a_1 x^2 + a_2 x^3). \tag{13}$$

There is a formal rather than a physical model behind Eq. (13). The fit quality obtainable with Eq. (13) is illustrated by the filled squares curve f Fig. 1.

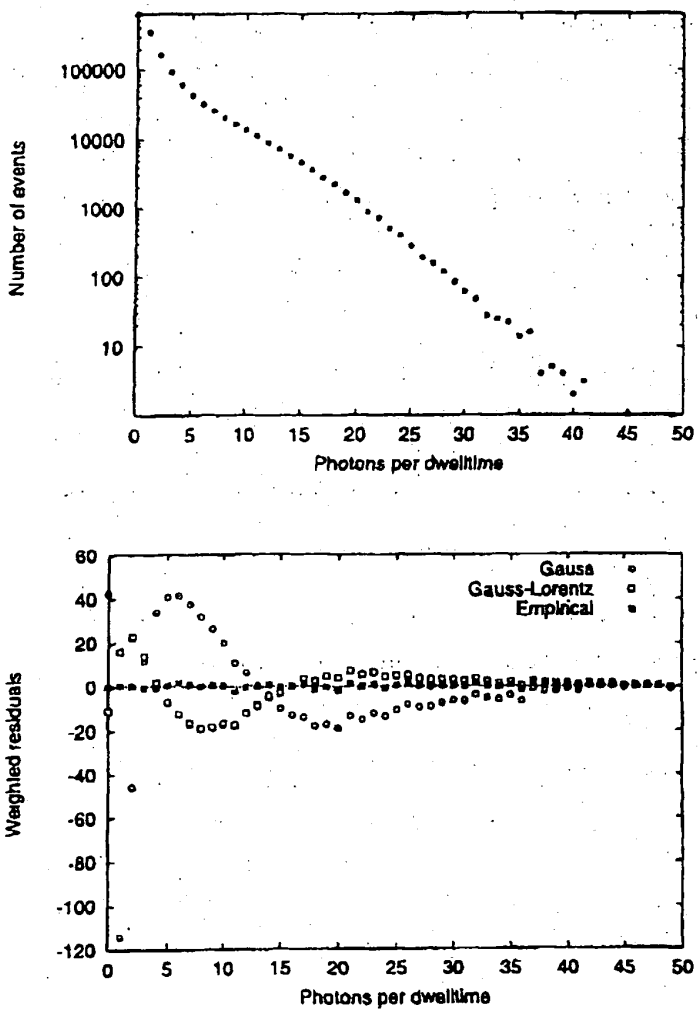

Fig. 1. A distribution of the number of photon counts measured at $T = 40$ μs for about 10^{-9} M solution of tetramethylrhodamine in water, data collection time 60 s. Three residuals curves are presented, corresponding to the best fit obtained with Eqs (11), (12), and (13).

6. Distribution of the specific brightness within a species

Some fluorescent species may have significantly wide distribution of specific brightness. For example vesicles, which are likely to have a significantly broad size distribution and a rand m number of receptors, may have trapped a random number of labeled ligand molecules. In order to fit count number distributions for samples containing such kind of species, we have modified Eq. (10) in the following manner. We assume that the distribution of brightness of particles q within a species is mathematically expressed as follows:

$$\rho(q) \propto q^{a-1}e^{-bq}. \tag{14}$$

This expression has been selected for the sake of convenience: all moments of this distribution can be analytically calculated, using the following formula:

$$\int_0^{\infty} x^a e^{-bx}dx = \frac{\Gamma(a+1)}{b^{a+1}}. \tag{15}$$

It is straightforward to derive the modified generating function of a photon count number distribution. Following the arguments of section 2.3 one can rewrite Eq. (9) as follows:

$$G(\xi) = \exp[\sum_i c_i \int dV \int_0^{\infty} dq \rho(q;a_i,b_i)(e^{(\xi-1)qTB(r)} - 1)], \tag{16}$$

where

$$\rho(q;a,b) = \frac{b^a}{\Gamma(a)} q^{a-1}e^{-bq}. \tag{17}$$

The integral over q can be performed analytically:

$$G(\xi) = \exp\left\{\sum_i c_i \int dV \left[\left(\frac{b_i}{b_i - (\xi-1)TB(x)}\right)^{a_i} - 1\right]\right\}. \tag{18}$$

The parameters a_i and b_i are related to the mean brightness $\bar{q}$ and the

width of the brightness distribution σ_i^2 by

$$a_i = \frac{\bar{q}_i^2}{\sigma_i^2}, \qquad b_i = \frac{\bar{q}_i}{\sigma_i^2}. \tag{19}$$

7. Weighting in FIDA

In the range of obtained count numbers, the probability to obtain a particular count number usually varies by many orders of magnitude, see for example the distribution of Fig. 1. Consequently, the variance of the number of events with a given count number has a strong dependence on the count number. To determine weights for least squares fitting, let us assume for simplification that light intensities in all counting intervals are independent. Under this assumption, we have a problem of distributing M events over choices of different count numbers n, each particular outcome having a given probability of realization, $P(n)$. Covariance matrix elements of the distribution can be expressed as follows:

$$\langle \Delta P(n) \Delta P(m) \rangle = \frac{P(n)\delta(n,m) - P(n)P(m)}{M}, \tag{20}$$

where M is the number of counting intervals per experiment.

For a further simplification, one may ignore the second term on the right side of Eq. (20), which can be interpreted as a consequence of normalization (see Appendix). In this case, the weights simply equal to the inverse values of the diagonal covariance matrix elements

$$W_n = \frac{M}{P(n)}. \tag{21}$$

8. Data simulation algorithms

Data simulation is a convenient means of testing FIDA algorithms and debugging the corresponding computer programs. As an illustration, in Fig. 2, a count number distribution is simulated for a case which models a binding reaction of a labeled ligand to vesicles. Distribution with open circles corresponds to the free ligand alone; distribution with open

squares corresponds to labeled vesicles; and distributi n with filled squares corresponds to their mixture. Concentrations and specific brightness values have been selected to model a realistic situation in drug screening.

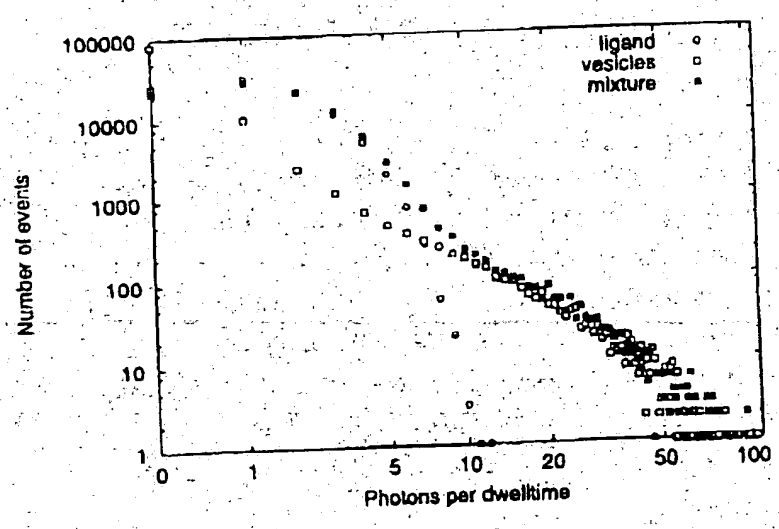

Fig. 2. Simulated distributions of the number of counts corresponding to $T = 40$ μs, values of the spatial parameters of Eq. (13) $a_1 = -0.4$; $a_2 = 0.08$, background count rate $b = 1.0$ kHz, data collection time 4 s. Curve „ligand" corresponds to a species of $c = 6.0$; $q = 6.0$ kHz/particle; $\sigma_q = 0$. Curve „vesicles" corresponds to a species of $c = 0.05$; $q = 300.0$ kHz/particle; $\sigma_q = 150.0$. Curve „mixture" corresponds to their mixture. Fitting of curve (c) returns the values of the five parameters characterizing the given „sample" with statistical errors which are mostly between 3.5 and 6 percent, except the error of σ_q of vesicles which is 13 percent. If, however, σ_q of vesicles is fixed in fitting, all the statistical errors are below 4 percent.

For the fastest data simulation algorithm, we calculate the expected distribution and generate a random Poisson number of events for each value of n independently.[vii] As a cosmetic error, the total number of events $\sum_n S(n)$ may slightly deviate from the pregiven number M.

A slower but a straightforward data simulation algorithm is the generation of a random configuration of particles in volume elements contributing to fluorescence, the calculation of the classical light intensity corresponding to the given configuration of particles, and the generation of a random Poisson number corresponding to this intensity, as a simulated number

of photon counts. This procedure is repeated M times to obtain a simulated count number distribution.

9. Statistical errors of estimated parameters

In general, a linear or linearized least squares fitting returns not only the values of the estimated parameters, but also their covariance matrix, provided the weights have been meaningfully set. It may turn out to be possible to express the statistical errors of the estimated parameters analytically in some simple cases (e.g., for the rectangular sample profile and single species) but in applications at least two-component analysis is usually of interest. Therefore, we have been satisfied with the numerical calculations of statistical errors. In addition to the „theoretical" errors with the assumption of uncorrelated measurements (Eq. (20)), in some cases we have determined statistical errors empirically, making a series of about a hundred FIDA experiments on identical conditions. As a rule, empirical errors are higher than theoretical ones by a factor of three to four. Empirical errors appear to be closer to the theoretical ones in scanning experiments. Therefore we are convinced that the main reason of the underestimation of theoretical errors is the assumption of uncorrelated measurements.

Figs. 3-8 illustrate how theoretical errors depend on the counting interval T, concentrations and brightness values. Table 1 compares statistical errors of parameters estimated by fitting a photon count number distribution (FIDA) and by the moment analysis. FIDA is overwhelmingly better than the moment analysis if the number if estimated parameters is higher than three.

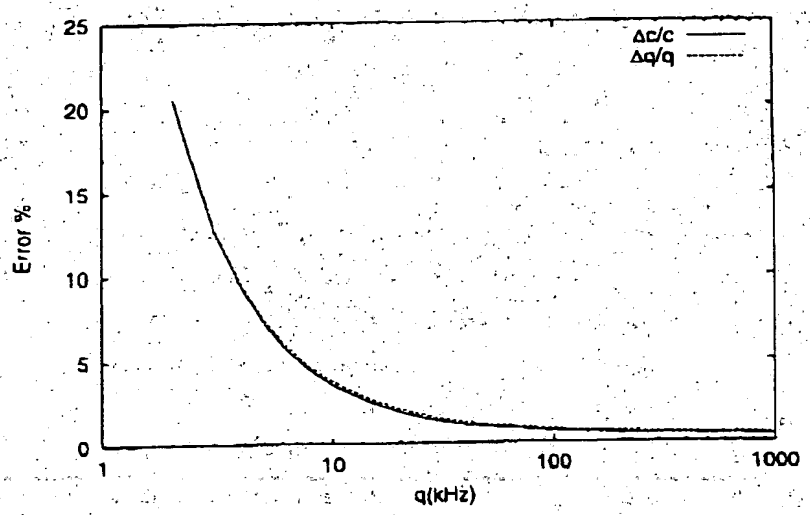

Fig. 3. Theoretical errors of the estimated parameters c and q of a solution of single species, depending on the value of q. The following values of experimental parameters were selected: $c = 1.0$; $T = 20$ μs; $a_1 = -0.4$; $a_2 = 0.08$; $b = 1.0$ kHz; data collection time 2 s.

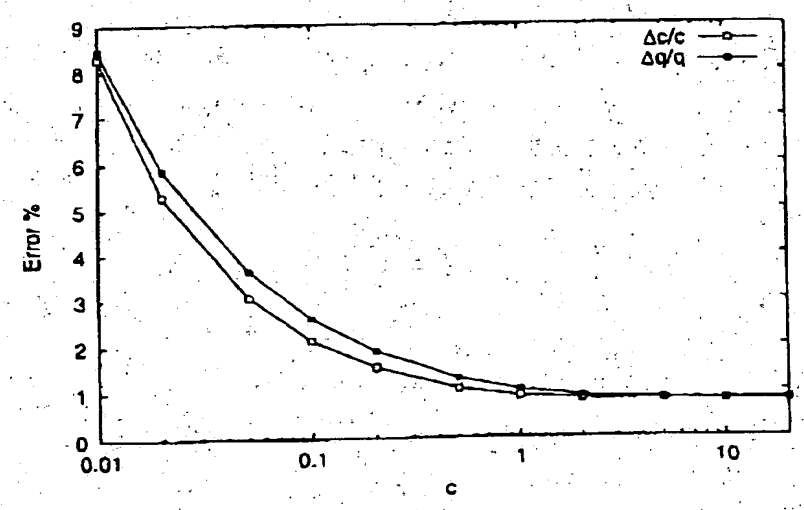

Fig. 4. Theoretical errors of the estimated parameters c and q of a solution of single species, depending on the value of c. The following values of experimental parameters were selected: $q = 60$ kHz/particle; $T = 20$ μs; $a_1 = -0.4$; $a_2 = 0.08$; $b = 1.0$ kHz; data collection time 2 s.

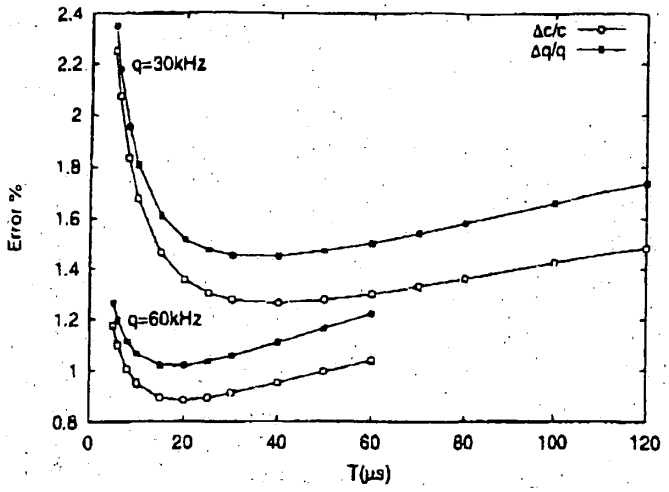

Fig. 5. Theoretical errors of the estimated parameters c and q of a solution of single species, depending on the value of T. The following values of experimental parameters were selected: $c = 1.0$; $q = 30.0$ kHz/particle (upper graphs); $q = 60.0$ kHz/particle (lower graphs); $a_1 = -0.4$; $a_2 = 0.08$; $b = 1.0$ kHz; data collection time 2 s.

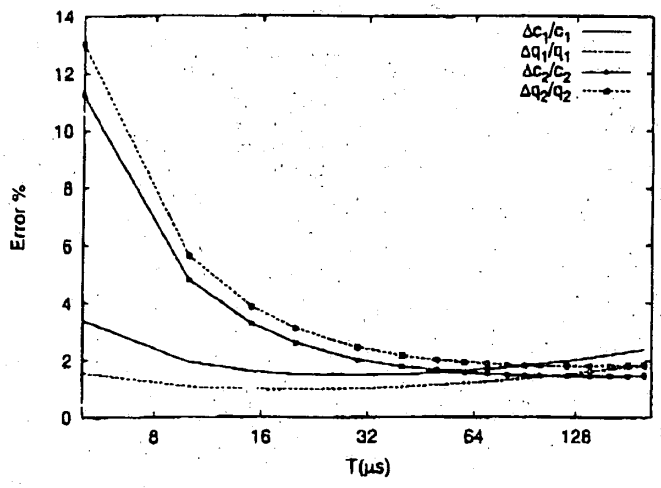

Fig. 6. Theoretical errors of the estimated parameters c and q of a mixture of two species, depending on the value of T. The following values of experimental parameters were selected: $c_1 = 0.1$; $c_2 = 2.0$; $q_1 = 200.0$ kHz/particle; $q_2 = 10.0$ kHz/particle; $a_1 = -0.4$; $a_2 = 0.08$; $b = 1.0$ kHz; data collection time 10 s. Note that the optimal value of T for the determination of the parameters of the brighter species is lower than that of the darker species.

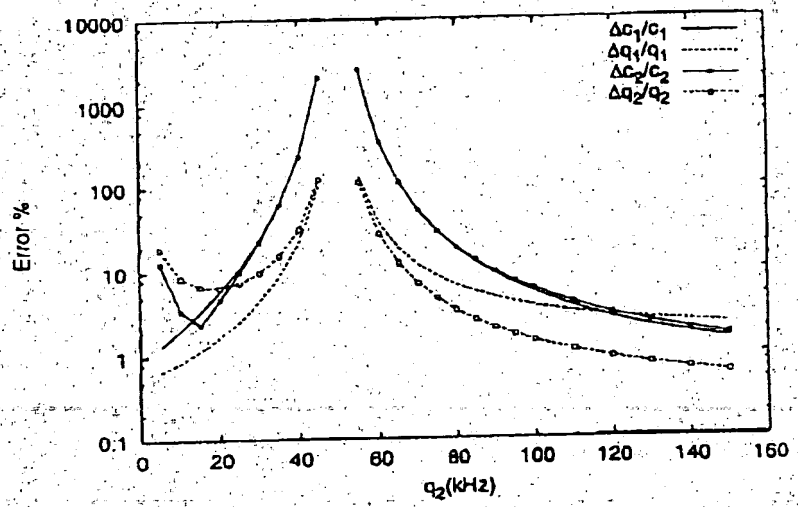

Fig. 7. Theoretical errors of the estimated parameters c and q of a mixture of two species, depending on the ratio of q_2 to q_1. The following values of experimental parameters were selected: q_1 = 50.0 kHz/particle; $c_1 = c_2 = 0.5$; a_1 = -0.4; a_2 = 0.08; b = 1.0 kHz; data collection time 40 s.

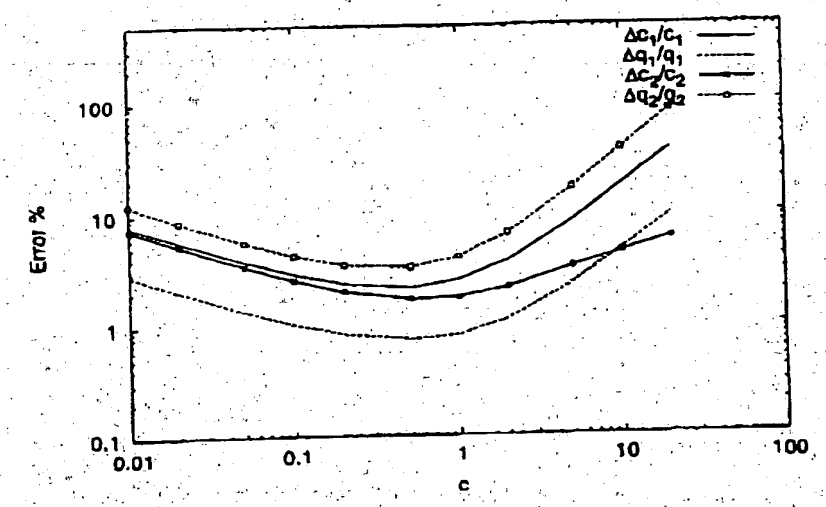

Fig. 8. Theoretical errors of the estimated parameters c and q of a mixture of two species, depending on concentrations. The concentrations were changed synchronously, $c_1 = c_2$. The

following values of experimental parameters were selected: q_1 = 75.0 kHz/particle; q_2 = 25.0 kHz/particle; a_1 = -0.4; a_2 = 0.08; b = 1.0 kHz; data collection time 60 s. Note that an optimal concentration exists at about one particle per sample volume. This is generally true, except if less than three parameters are to be determined.

Data collection time, s	Time window, μs	Number of species	Number of estimated parameters	Parameter specification	Value (qs in kHz)	Percent error of FIDA	Percent error of moment analysis
10.0	40.0	1	2	c	0.5	0.59	0.54
				q	60.0	0.56	0.51
10.0	40.0	2	3	c_1	0.05	2.00	2.71
				q_1	150.0	1.54	1.89
				c_2	3.0	0.53	0.62
				q_2	5.0 (fixed)		
10.0	40.0	2	4	c_1	0.05	2.26	4.99
				q_1	150.0	1.63	2.53
				c_2	3.0	3.18	17.8
				q_2	5.0	3.35	14.9

Table 1. Statistical errors of estimated parameters from least squares fitting of photon count number distributions (FIDA) and from moment analysis. Error values are determined through processing a series of simulated distributions.

Appendix

Dispersion matrix (20) corresponds to the multinomial distribution of statistical realizations of histograms. We will show here that the Poissonian distribution, with the constraint that the total number of counting intervals M is fixed, will lead to the multinomial distribution. This is the rationale behind using Poissonian weights as given in Eq. (21).

Let n_k be the expectation value of the number of events of counting k photons and let $N = \sum_k n_k$ be their sum. Let m_k be a statistical realization with $M = \sum_k m_k$. Assume that realizations $m_0, m_1, \ldots$ obey Poissonian statistics

$$P(m_0, m_1, \ldots) = \frac{[n_0, n_1, \ldots]^{\{m_0, m_1, \ldots\}}}{[m_0, m_1, \ldots]!} e^{-N}, \qquad \text{(A1)}$$

where we hav introduced the notation $n_0^{m_0} n_1^{m_1} \ldots \equiv [n_0, n_1, \ldots]^{[m_0, m_1, \ldots]}$. The probability of having the total of M events is

$$P(M) = \frac{N^M}{N!} e^{-N}. \qquad \text{(A2)}$$

The conditional probability of having $m_0, m_1, \ldots$ events if there is a total of M events is

$$P(m_0, n_1, \ldots | M) \equiv \frac{P(m_0, m_1, \ldots)}{P(M)} = \frac{M![n_0, n_1, \ldots]^{[m_0, m_1, \ldots]}}{N^M [m_0, m_1, \ldots]!},$$

or

$$P(m_0, m_1, \ldots | M) \equiv C_{m_0, m_1, \ldots}^{M} [p_0, p_1, \ldots]^{[m_0, m_1, \ldots]}. \qquad \text{(A3)}$$

This is the multinomial distribution where we have introduced:

$$p_k \equiv \frac{n_k}{M}$$

and $C_{m_0, m_1, \ldots}^{M}$ are multinomial coefficients.

---

[i] H. Qian and E. L. Elson, Biophys. J. 57: 375-380 (1990).

[ii] Under 'specific brightness' we mean count rate of the detector from light emitted by a particle of given species situated in a certain point in the sample, conventionally in the point where the value of the spatial brightness profile function is unity.

[iii] H. Qian and E. L. Elson, Proc. Natl. Acad. Sci. USA. 87: 5479-5483 (1990).

[iv] Usually in FCS, the unit of volume and the unit of B are selected which yield $\chi_1 = \chi_2 = 1$. After selecting this convention, concentrations in our equations are dimensionless, expressing the mean number of particles per „sample volume", and the specific brightness of any species equals the mean count rate from a particle if situated in the focus divided by the numeric value of $B(0)$. The value of this constant is a characteristic of optical equipment. It can be calculated from estimated parameters of the spatial intensity profile (see section 2.4); it is about 3.8 for our equipment.

[v] P. Kask, K. Palo, D. Ullmann, and K. Gall, Fluorescence Intensity Distribution Analysis and its Application in Biomolecular Detection Technology (a manuscript presented to PNAS).

[vi] Indeed, moments of the three-dimensional Gaussian profile do not depend on axial dimension ratios but only on their product which is an absolute measure of the sample volume.

[**] Generation of a random Poisson number is the following. For a given expected value of events E, a simulated number of events S is determined from a routinely generated random number R between 0.0 and 1.0 through inequations $\sum_{i=0}^{S-1} Poisson(i;E) < R \le \sum_{i=0}^{S} Poisson(i;E)$.